HISTONE DEACETYLASE INHIBITOR TRICHOSTATIN A SUSTAINS 
SODIUM PERVERANADATE-INDUCED NF-κB ACTIVATION BY 
DELAYING IkBα mRNA RESYNTHESIS - 
COMPARISON WITH TNFα

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Abbreviations: ALLN, N-acetylleucylleucylnorleucinal; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; ERK1/2, extracellular signal-regulated kinase 1/2; HAT, histone acetyltransferase; HDAC, histone deacetylase; HIV LTR, HIV long terminal repeat; H2O2, hydrogen peroxide; IKK, IkB kinase; MAPK, mitogen-activated protein kinase; MSK1, mitogen- and stress-activated protein kinase-1; NF-κB, nuclear factor kappa B; NT, non treated; P-, phosphorylated residue; PMA, phorbol myristate acetate; PTPase, protein tyrosine phosphatase; PV, sodium pervanadate; qRT-PCR, quantitative real-time reverse transcriptase-PCR; RNA Pol II, RNA polymerase II; RPA, ribonuclease protection assay ; SAHA, suberoylanilide hydroxamic acid; TNFα, tumor necrosis factor alpha; TSA, trichostatin A.

Running title: Delay of PV-induced IkBa mRNA synthesis by TSA addition

Key words: NF-κB, trichostatin A, pervanadate, IkBα synthesis, histone H3, IKKα

SUMMARY

NF-κB is a crucial transcription factor tightly regulated by protein interactions and post-translational modifications, like phosphorylation and acetylation. A previous study has shown that trichostatin A (TSA), an histone deacetylase (HDAC) inhibitor, potentiates TNFα-elicited NF-κB activation and delays IkBα cytoplasmic reappearance. Here, we demonstrated that TSA also prolongs NF-κB when induced by the insulino-mimetic pervanadate (PV), a tyrosine phosphatase inhibitor which initiates an atypical NF-κB signaling. This extension is similarly correlated with delayed IkBα cytoplasmic reappearance. However, whereas TSA causes a prolonged IKK activity when added to TNFα, it does not when added to PV. Instead, qRT-PCR revealed a decrease of ikba mRNA level after TSA addition to PV stimulation. This synthesis deficit of the inhibitor could explain the sustained NF-κB residence in the nucleus. In vivo analysis by ChIP assays uncovered that, for PV induction but not for TNFα, the presence of TSA provokes several impairments on the ikba promoter : (i) diminution of RNA Pol II recruitment; (ii) reduced acetylation and phosphorylation of histone H3-Lys14 and -Ser10, respectively; (iii) decreased presence of phosphorylated p65-Ser536; and (iv) reduction of IKKα binding. The recruitment of these proteins on the icam-1 promoter, another NF-κB-regulated gene, is not equally affected, suggesting a promoter specificity of PV with TSA stimulation. Taken together, these data suggest that TSA acts differently depending on the NF-κB pathway and the targeted promoter in question. This indicates that one overall HDAC role is to inhibit NF-κB activation by molecular mechanisms specific of the stimulus and the promoter.
INTRODUCTION

The ubiquitous nuclear factor (NF)-κB is a critical regulator of the expression of numerous genes implicated in immune and inflammatory responses, cellular proliferation and differentiation and cell survival (1). This transcription factor is composed of homo- or heterodimers with various combinations of five subunits: p50/p105, p52/p100, p65 (RelA), RelB and c-Rel. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through its association with a member of an inhibitory family, of which the most characterized is IκBα (2). Upon cell stimulation by tumor necrosis factor alpha (TNFα), inducing the classical pathway, IκBα is rapidly phosphorylated on Ser32 and -36 by the cytoplasmic IκB kinase (IKK) complex, which triggers its polyubiquitination and subsequent degradation. The released NF-κB translocates into the nucleus to regulate the expression of multiple target genes, including those coding for its own inhibitor, IκBα. This negative feed-back ensures removing NF-κB from its DNA-binding sites and transporting it back to the cytoplasm, thereby terminating NF-κB-dependent transcription (3).

Tyrosine phosphorylation plays a key role in NF-κB activation. It has been shown that pervanadate (PV), a potent tyrosine phosphatase (PTPase) inhibitor, induces IκBα phosphorylation on Tyr42 and activates NF-κB (4). Depending on the cell type, this IκBα tyrosine phosphorylation is correlated with either dissociation from NF-κB (5) or IκBα degradation (6). In Jurkat T cells stimulated with PV, the phosphatidylinositol 3-kinase (PI3K) regulatory subunit, p85α, interacts with the Tyr-phosphorylated IκBα, leading to IκBα release from NF-κB without its degradation (7). In HeLa cells, PV as well as hypoxia/reoxygenation, causes Tyr-phosphorylation of IκBα through a Src-dependent mechanism (8). Beside this, PV is also considered as an insulin-mimetic compound by its capacity to activate insulin tyrosine kinase receptor and mediate the insulin metabolic actions through activation of extracellular signal-regulated kinase 1/2 (ERK1/2), PI3K and protein kinase B (PKB/Akt) (9).

Acetylation is a pivotal post-translational modification of numerous proteins, such as histones and transcription factors (like NF-κB). Histone acetylation, required for transcriptional activation, is tightly controlled by histone acetyltransferases (HAT) and histone deacetylases (HDAC). Several cancer-promoting mutations result in repression of transcription through abnormal recruitment and activation of HDAC that may lead to neoplastic transformation. Therefore, HDAC inhibitors have emerged as new agents for cancer treatment by preventing angiogenesis and inducing growth arrest with a remarkable tumour specificity (10,11).

NF-κB functions are regulated by post-translational modifications including phosphorylation (12,13) and acetylation (14). These modifications determine both the strength and duration of NF-κB-mediated transcriptional response (15). Among the numerous residues emerging to be phosphorylated on p65, Ser276 and Ser536 are the best characterized. Phosphorylation of Ser276 is mediated by protein kinase A catalytic subunit (PKAε) or mitogen- and stress activated kinase 1 (MSK1) (16,17) and Ser536 is targeted by multiple kinases : IKKα, IKKB, IKKe and TRAF family member-associated (TANK)-binding kinase (TBK1) (18,19). These phosphorylations allow Lys310 acetylation by CBP/p300 and transcriptional activation (20). Importantly, p65 phosphorylation status determines whether nuclear NF-κB associates with CBP/p300 or HDAC, leading to p65 transcriptional activation or repression, respectively (21-23).

A previous study has shown a potentiation of TNFα-induced NF-κB activation by deacetylase inhibitors (24). The associated cytoplasmic reappearance of IκBα is delayed, which is explained, at least partially, by a prolonged IKK activity. Here we analyzed the influence of histone deacetylase inhibitor on a NF-κB pathway involving tyrosine phosphorylations induced by PV. We demonstrated that the HDAC inhibitor TSA extends the PV-induced NF-κB activation by a distinct mechanism. Indeed, iκba mRNA synthesis appears to be delayed after the co-stimulation with PV and TSA, explaining the NF-κB persistence in the nucleus of treated cells. This delay of iκba mRNA synthesis seems to be due to impairing of the recruitments of IKKα, p65 phosphorylated on Ser536 and RNA Pol II, but also acetylation of histone H3 on Lys14, and phosphorylation of histone H3 on Ser10. This extension of NF-κB...
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activation is completely different from the one initiated by TNFα with TSA which is due to, at least partly, an extension of the IKK activity. Therefore, we show here that the implication of HDAC inhibitors can be quite different depending on the NF-κB-inducing agent.

EXPERIMENTAL PROCEDURES

Cell lines and reagents - HeLa cells were cultured in EMEM with 10% foetal calf serum and glutamine (Biowhittaker, Petit Rechain, Belgium). Jurkat T cells were cultured in RPMI 1640 with 10% foetal calf serum and glutamine (Biowhittaker). TSA was used at the concentration of 450 nM (Sigma, St Louis, MI, USA), TNFα at 200 U/mL (Roche, Mannheim, Germany) and SAHA at 3µM (Alexis Biochemicals, Zandhoven, Belgium). Sodium pervanadate (PV) was freshly prepared before each experiment as previously described (Imbert et al., 1996) and it was used at 200 µM. $H_2O_2$ treatment (250 µM; Sigma) was always preceded by a preincubation with aminotriazole (50mM, 1h) (Sigma), a catalase inhibitor (4). The phorbol ester PMA (200 nM) was always combined with the Ca$^2+$ ionophore ionomycine (141 nM) (Sigma). N-acetylleucylleucylnorleucinal (ALLN) was used at the concentration of 100 µM (Sigma).

Antibodies - Monoclonal anti-IκBα used for western blotting was provided by Dr. Ron Hay (University of Dundee, UK). Polyclonal anti-IκBα used for immunoprecipitation, -p65 used for western blot, -IKKβ, -RNA Pol II and -IKKα were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IκBα phosphorylated on Ser32 and -36, -phospho-p38 and -phospho-ERK, -p65 phosphorylated on Ser276 and -p65 phosphorylated on Ser536 were from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-tyrosine, -p65 used for immunoprecipitation, -histone H3 acetylated on Lys14 and -histone H3 phosphorylated on Ser10 were from Upstate (Charlottesville, VA, USA). Finally anti-unmodified histone H3 were from Abcam Limited (Cambridge, UK).

Plasmids - Several reporter plasmids containing the Luciferase gene were used under the control of different promoters were used. The 0.4SK-pGL3 plasmids containing the $iκb$a promoter were kindly provided by J. Hiscott (Mc Gill University, Canada). The plasmid picam-1-Luc was donated by Y. de Launoit (University of Brussels, Belgium).

Transient transfection and luciferase assay – Twenty hours before treatment, HeLa cells were transfected with FuGENE 6™ (Roche) according to the manufacturer’s recommended procedures. At 7h post-treatment, cells were lysed and assayed for luciferase activity. Luciferase activities were normalized with protein concentration. Luciferase assay results are an average of three independent experiments.

Cytoplasmic and nuclear protein extraction - Cells were washed twice with ice-cold PBS, scraped and centrifuged. The pellets were resuspended in 100 µL of cold hypotonic buffer (10 mM Hepes-KOH pH 7.9, 2 mM MgCl$_2$, 0.1 mM EDTA, 10 mM KCl, 0.5% IGEPAL, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail Complete (Roche)), incubated on ice for 10 min. The lysates were vortexed 5 sec, centrifuged for 30 sec at 14,000 rpm at 4°C. The supernatants containing cytoplasmic proteins were stored at -80°C. The pellets were next resuspended in 30 µL of cold hypertonic buffer (50 mM Hepes-KOH pH 7.9, 2 mM MgCl$_2$, 0.1 mM EDTA, 400 mM NaCl, 10% glycerol, 1 mM DTT, 0.5mM PMSF and Complete), incubated on ice for 25 min and centrifuged for 15 min at 14,000rpm at 4°C. Then the supernatants containing nuclear proteins were stored at -80°C. The protein concentration was determined with Bio-Rad protein assay (Hercules, CA, USA).

Western blotting and electrophoretic mobility shift assay (EMSA) – Cytoplasmic extracts, obtained as described above, were analyzed by western blotting as previously described (25). Nuclear extracts, prepared as detailed above, were analyzed by EMSA as previously described (26), using a $^{32}$P-labeled oligonucleotide probe (5’-GGTTACAAGGGACTTCCGCTG-3’; Eurogentec, Liège, Belgium) corresponding to a HIV-1 LTR κB site.

IκBα immunoprecipitation – Whole cell extraction and IκBα immunoprecipitation were previously described by Gloire et al. (27). The presence of unmodified IκBα and IκBα

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phosphorylated on tyrosine was determined by western blotting.

**IKK complex immunoprecipitation and in vitro IKK kinase assay** – Cytoplasmic extracts were prepared as detailed above in a hypotonic buffer supplemented with phosphatase inhibitors (1 mM Na₂VO₃, 10 mM NaF, 25 mM β-glycerophosphate, 10 mM nitrophenylphosphate). IKK complex immunoprecipitation and in vitro IKK kinase assay were performed as previously described (28) with purified GST-IκBα1-54 fusion protein as substrate (a gift from R. Gaynor UT Southwestern Medical Center at Dallas). This was followed by a western blotting using an anti-IκB phosphorylated on Ser32 and -36 antibody.

**Total protein extraction for phospho-western blotting** – Treated cells were washed with ice-cold PBS and rapidly lysed in SDS-blue lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.03% bromophenol blue powder and 50 mM DTT). Total lysates were sonicated for 50 sec, boiled for 3 min and used for western blotting as previously described (25).

**Quantitative Real-Time Reverse Transcription-PCR** - Total RNA samples were extracted with RNeasy Mini Kit (QIAGEN, CA, USA) according to manufacturer’s recommendations. 1 µg of RNA were submitted to reverse transcription with the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For quantitative real-time RT-PCR, the obtained cDNA was analyzed, in triplicate, with the SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) in the ABI Sequence Detection System. The results were normalized with the β2-microglobulin transcript. The primers used to analyze the different transcripts were designed with the software Primer Express™ (Applied Biosystems): iκbα, FW 5’-CCAACCCAGCCAGAAATTGCT-3’ and RV 5’-TTCGGGAGCTCAGGATACA-3’; icam-1, FW 5’-TGCCAAGAGGGGAGGCGTG-3’ and RV 5’-GCCCGGGAGGCTCCGTGC-3’; β2-microglobulin, FW 5’-GAGTATGCTGGCAGTTG-3’ and RV 5’-AATCCAAATGCGGCATCT-3’ (Eurogentec).

**Chromatin Immunoprecipitation Assay** – Chromatin immunoprecipitation (ChIP) assays were carried out with solutions prepared in our laboratory following the Upstate Cell Signaling protocol. Chromatin was sheared by sonication for 15 min to lengths between 200 and 1000 basepairs. The sonication was done in a water bath with generation of high power ultrasound (Bioruptor, Diagenode, Belgium). Chromatin immunoprecipitations were performed with 2 µg of different antibodies: anti-p65, -RNA Pol II, -histone H3 acetylated on Lys14, -histone H3 phosphorylated on Ser10, -p65 phosphorylated on Ser276, -p65 phosphorylated on Ser536 and -IKKα. To test aspecific binding to the beads, an irrelevant antibody was used as control for immunoprecipitation (anti-Flag antibody, Sigma). A phenol/chloroform DNA extraction was performed and the immunoprecipitated DNA was analyzed by quantitative real-time PCR with the SYBR Green Master Mix in the ABI Sequence Detection System. All ChIP assays were performed three times. The primers, corresponding to the promoter region of each gene, were designed using the software Primer Express™:

- iκbα, FW 5’-CGCTCATCAAAAAAGTCCCTG-3’ and RV 5’-GGATTTCAAGGCCAGTCGAC-3’
- icam-1, FW 5’-CCCGATTGCTTTAGCTTGGAA-3’ and RV 5’-CCGGAAACAAATGCTGCAAT-3’ (Eurogentec).

**RESULTS**

It was previously reported by Adam et al. (24) that histone deacetylase inhibitors cause the extension of TNFα-induced NF-κB activation when both compounds are added simultaneously. In this work, we compared the influence of trichostatin A (TSA), a large spectrum histone deacetylase inhibitor, on two different NF-κB activation pathways: (i) the atypical pathway involving multiple tyrosine kinases mediated either by sodium pervanadate
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(PV) or hydrogen peroxide (H$_2$O$_2$), (ii) the classical pathway induced either by the pro-inflammatory cytokine TNFα or the phorbol ester PMA.

**Influence of HDAC inhibitor on NF-κB activation, and the associated IκBα degradation, induced by various inducers** – We investigated the kinetics of NF-κB activation elicited by four inducers (PV, TNFα, PMA and H$_2$O$_2$) in presence or absence of an HDAC inhibitor, trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA), in two cell types, HeLa cells or Jurkat T cells. Time course extractions were carried out after each co-treatment. Electromobility shift assays (EMSA), performed with nuclear extracts, revealed that stimulation with PV alone leads to NF-κB activation from 30 min until 2h (Fig.1A, upper left panel), whereas the co-treatment PV plus TSA potentiates this binding until 8h (Fig.1A, upper middle panel). The associated IκBα degradation in the corresponding cytoplasmic extracts was analyzed by western blotting. A delay in the IκBα cytoplasmic reappearance is clearly detected when TSA is added simultaneously to PV (Fig.1A, lower left and middle panels). Beside this, we tested what happen to the PV-induced NF-κB activation with either another HDAC inhibitor (SAHA) or another cell type (Jurkat T cells). First, in HeLa cells, we observed by EMSA that the simultaneous addition of SAHA also prolongs the activation of NF-κB induced by PV until 8h (Fig.1A, upper right panel), which is correlated with a delay of IκBα cytoplasmic reappearance shown by western blotting (Fig.1A, lower right panel). TSA and SAHA giving similar results, SAHA impacts are not investigated anymore in this report. In Jurkat T cells, Beraud et al. (7) have demonstrated that PV induces NF-κB activation via an IκBα phosphorylation on tyrosine associated with its dissociation from NF-κB. Here in this cell type, we detected no significant extension of NF-κB activation when TSA is added (Fig.1B, upper panels), but the IκBα phosphorylation, associated with NF-κB activation, is prolonged from 30 min to, at least, 1h (Fig.1B, lower panels). This indicates a sustained activity of IκBα tyrosine kinase. Thus, the use of Jurkat T cells highlights the fact that the intensity of the HDAC inhibitor effects on NF-κB activation could depend on the cell type.

The following experiments were performed with the HDAC inhibitor TSA on HeLa cells, for which the effects are the most striking.

The events obtained after treatment of HeLa cells with PV in absence or in presence of TSA seem to be similar to those described by Adam et al. (24) and are confirmed here, with HeLa cells treated with TNFα and TSA compared to cells treated with TNFα alone. We observed NF-κB activation from 10 min to 30 min with TNFα alone, whereas the addition of TSA extends it until 4h. The IκBα cytoplasmic reappearance is also delayed for several hours (Fig.1C). The induction by the phorbol ester PMA, leading to NF-κB activation via PKC and IKK complex (30), also shows a prolonged profile when TSA is added ; the kinetic is however rather different (Fig.1D). In HeLa cells, H$_2$O$_2$ was described by Storz et al. to activate the IKK complex but fail to induce IκBα tyrosine phosphorylation (31). In this study, H$_2$O$_2$ is the only inducer that shows no prolonged NF-κB activation upon simultaneous induction with TSA (Fig.1E). Nevertheless, the binding seems to be stronger at 4h of co-treatment. IκBα does not appear to be significantly degraded after H$_2$O$_2$ or H$_2$O$_2$ plus TSA stimulation. Therefore, TSA provokes the extension of NF-κB activation and delays IκBα reappearance in the cytoplasm in PV-, TNFα- and PMA- induced pathways, whereas the NF-κB activation by H$_2$O$_2$ is not significantly modified by the presence of TSA.

**Effect of TSA on IκBα tyrosine phosphorylation or on IKK activity after stimulation by PV, TNFα, PMA or H$_2$O$_2$** – In order to understand the origin of this prolonged NF-κB activation, we focused on the events responsible for IκBα phosphorylation and its subsequent degradation. PV is known to induce global tyrosine phosphorylation of proteins by its ability to inhibit tyrosine phosphatases. IκBα, one of the targets of tyrosine kinases, is phosphorylated on Tyr42 upon PV stimulation (5). Thus, we wanted to explore whether this tyrosine phosphorylation is influenced by the addition of TSA. HeLa cells were pretreated for 45 min with a proteasome inhibitor, ALLN, to protect phosphorylated IκBα from degradation, and then treated with PV with or without TSA for 30 min or 2h. Total extracts were used to immunoprecipitate IκBα and its
phosphorylation status on tyrosine was determined by western blotting with an anti-phospho-Tyr antibody (Fig.2A, upper panel). When we compare the level of tyrosine phosphorylation 30 min after PV stimulation without and with TSA, there is no obvious difference. Identical results are obtained after 2h of treatment. The amount of immunoprecipitated IκBα, visualized using an anti-IκBα antibody, is quite similar in the samples without and with TSA at each time point, even if the total level of IκBα is slightly decreased after 2h (Fig.2A, lower panel).

IKK activity, that is responsible for IκBα phosphorylation on Ser32 and -36, was then tested after exposure to PV with or without TSA for periods ranging from 5 min to 4 h. A treatment with TNFα for 5 min was used as a positive control. The IKK complex was immunoprecipitated from cytoplasmic extracts and submitted to an in vitro kinase assay with purified GST-IκBα1-54 fusion protein as substrate. A western blotting was then performed with an antibody recognizing IκBα phosphorylated on Ser32 and -36. We observed that the PV- and H2O2- induced IKK activity, already shown in HeLa cells by Storz et al. (31), is weaker than the one observed with TNFα and is not considerably affected, or even slightly decreased, by the presence of TSA (Fig.2B and 2C respectively, upper panels). On the opposite, when TSA is added to TNFα or PMA, the IKK activity is prolonged, respectively up to 2h or 4h of treatment (Fig.2D, upper panels). The quality of the immunoprecipitation is visualised with an anti-IKKβ antibody in each IKK kinase assay (Fig.2B, 2C and 2D, lower panels).

Taken together, these results indicate that TSA has no effect upstream of PV- and H2O2- elicited IκBα degradation, whereas there is an extension of TNFα- and PMA-induced IKK activity by the addition of TSA.

For the following results, we focused on the comparison of the two inducers, PV and TNFα. PMA stimulation is not further considered as it displays the same profile than TNFα when TSA is added. Likewise, since TSA has only a slight influence on H2O2 induction in these experiments, we decided not to further studied this rather modest effect.

**PV decreases PV-, but not TNFα-, induced iκbα mRNA expression** – Because the delayed IκBα cytoplasmic reappearance after PV plus TSA treatment is not due to a prolonged phosphorylation and subsequent degradation, we investigated the iκbα mRNA synthesis. HeLa cells were treated with either PV or TNFα with or without TSA and total RNA was isolated. iκbα specific primers were used to examine RNA by quantitative real-time RT-PCR. The results were normalized with the β2-microglobulin transcript. When cells are treated for 1h with PV alone, a 3-fold stimulation of the iκbα mRNA synthesis is observed and is gradually decreased at 2h and 4h of treatment (Fig.3A, black bars). When TSA is added to PV, there is a strong reduction of iκbα mRNA synthesis at 1h and 2h of treatment, bringing it to levels quite comparable to the non treated signal (Fig.3A, grey bars). This effect of TSA on PV-induced iκbα mRNA expression seems to be stabilized at 4h, where we detected a slightly delayed resynthesis. This experiment was confirmed by Ribonuclease Protection Assay (RPA) (data not shown). In contrast, no significant difference appears between TNFα or TNFα plus TSA (Fig.3B), as it was previously described by RPA (24). Therefore, these results demonstrate that iκbα mRNA synthesis is delayed after the addition of TSA to PV, but not after the addition of TSA to TNFα. This reduction might explain why the nuclear presence of NF-κB is prolonged, as in the absence of newly synthesized inhibitor, NF-κB cannot be brought back to the cytoplasm.

**PV-induced NF-κB transcriptional activity on the iκbα promoter is reduced in presence of TSA** – In order to clarify the functional role of the NF-κB-binding site on iκbα promoter inducibility by PV, transient transfection assays were performed with a luciferase reporter gene under the control of the iκbα promoter (32). The addition of TSA decreases nearly 10-fold the PV-induced NF-κB transcriptional activity, while there is no significant difference of TNFα-elicited NF-κB transcriptional activity in presence of TSA (Fig.3C, logarithmic scale). Thus, NF-κB transcriptional activity on the iκbα promoter is clearly modified when TSA is combined with PV.

**The effect of TSA on PV induction is promoter-dependent** – In order to determine whether this TSA effect on the activity of the IκBα is observed with other NF-κB-dependent
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promoters, several promoters (il-8, il-6 and icam-1) were tested and the icam-1 promoter (33) showed interestingly different results. HeLa cells were treated with either PV or TNFα with or without TSA and total RNA was isolated. icam-1 gene specific primers were used to analyze RNA by quantitative real-time RT-PCR. The results were normalized with the β2-microglobulin transcript. The comparison between PV and PV plus TSA stimulation revealed no significant difference of icam-1 mRNA synthesis (Fig.4A). Unexpectedly, the addition of TSA to TNFα initiates a 2-fold decrease of icam-1 mRNA expression after 1h, which is stabilized and even slightly up-regulated at 4h (Fig.4B). This effect is quite similar to the one obtained after PV with or without TSA on the iκba promoter.

We also examined the functional role of NF-κB binding site in the inducibility of icam-1 promoter. Transient transfection assays were performed with a luciferase reporter gene under the control of the icam-1 promoter (Fig.4C). For both PV and TNFα inductions, the presence of TSA does not lead to significant change of NF-κB driven transcription. These results indicate that the icam-1 promoter seems to react differently than the iκba promoter after a co-stimulation of PV plus TSA. In summary, the effect of TSA on PV or TNFα induction is clearly promoter-dependent.

**Impact of TSA on p65 and RNA Pol II recruitments and histone H3 modifications on the iκba promoter after PV or TNFα induction** – In the previous paragraph, we showed that NF-κB, present in the nucleus of the co-treated (PV with TSA) cells, is able to bind longer to an in vitro probe by EMSA (Fig.1A), while iκba mRNA expression is impaired (Fig.3A). We thus investigated, by chromatin immunoprecipitation assays (ChIP), in vivo p65 and RNA Pol II recruitments, as well as histone H3 modifications, on the iκba promoter. Whatever the stimulus, PV or TNFα, the approximate 3-fold increase in p65 binding is not significantly modified by the presence of TSA (Fig.5A). As p65 is correctly recruited to the iκba promoter but mRNA synthesis is impaired, we next decided to analyze the RNA Pol II recruitment on the iκba promoter. At 15 min post treatment, the slight PV-induced RNA Pol II binding is not significantly modified by TSA, whereas, after 30 min, the addition of TSA diminishes the 3-fold increased recruitment by about 40%. At longer time, after one hour, the difference is not significant anymore. When we compared TNFα alone and TNFα plus TSA, there is no change of RNA Pol II binding (Fig.5B). It is commonly accepted that histone acetylation is a prerequisite to basal transcriptional machinery recruitment (34). Beside this, Yamamoto et al. have demonstrated that histone H3 must be phosphorylated on Ser10 prior to be acetylated on Lys14 on the iκba promoter (35). Therefore, in vivo histone H3 acetylation and phosphorylation were tested by ChIP assays and were normalized with unmodified histone H3 (Fig.5C and 5D). We noticed that histone H3 acetylation on Lys14 and phosphorylation on Ser10 are significantly down-regulated after 15 and 30 min of PV plus TSA treatment compared to PV alone. After 1h, the levels are approximately similar. However, when cells were treated with TNFα, TSA addition leads to an increase of both post-translational modifications on the iκba promoter. For each experiment, negative controls were performed with irrelevant immunoglobulins (Flag antibody) for immunoprecipitation and quantitative PCR on albumin promoter (data not shown). In conclusion, the impairment of iκba mRNA expression induced by the co-stimulation of PV and TSA is likely to be due to, at least partially, a delay of RNA Pol II recruitment and of histone H3 acetylation on Lys14 and phosphorylation on Ser10. The results are obviously different in presence of another inducer, TNFα.

**Impact of TSA on p65 and RNA Pol II recruitment and histone H3 modifications on the icam-1 promoter after PV or TNFα induction** – Since the influence of TSA on NF-κB-responsive genes appears to depend on the considered promoter, we next explored the icam-1 promoter and its in vivo p65/RNA Pol II recruitments and histone H3 modifications by ChIP assay. Whatever the inducer, PV or TNFα, neither p65 nor RNA Pol II recruitment is affected when TSA is added simultaneously (Fig.6A and B). For histone H3 acetylation on Lys14, TSA causes an up-regulation of the PV induction, particularly at 1h, but has no significant effect on the TNFα stimulation (Fig.6C). Interestingly, histone H3 phosphorylation on Ser10 is increased after
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TSA addition on PV stimulation as early as 15 min and that up-regulation is maintained up to 1h. The opposite is observed with TNFα as a diminution of histone H3 phosphorylation is induced by the presence of TSA (Fig.6D). This indicates that TSA differentially affects *ixba* and *icam-1* promoter regulation in a stimulus-specific mode.

The MAPK activation pathway remains unchanged after adding TSA to PV induction – MSK1, activated by ERK and p38 MAPK (36), is responsible for the phosphorylation of p65 on Ser276 (17) and H3 histone on Ser10 (37). Since H3 histone phosphorylation is reduced on the *ixba* promoter when TSA is added to PV stimulation (15 and 30 min) (Fig.5D), the MAPK activation pathway was analyzed by western blotting on total protein extracts. No change in ERK and p38 activation is detected, using phosphorylated antibodies, when cells are treated with PV in absence or presence of TSA (Fig.7A). The global phosphorylation status of two MSK1 targets, histone H3 and p65, were also checked after PV treatment and demonstrated that TSA addition has no impact on it (Fig.7B). The elevated level of histone H3 phosphorylation in the untreated cells can be explained by the fact that this phosphorylation occurs after cellular stress or mitogenic stimulation, as well as during mitosis. For the latter, MSK1 is not involved (37). As histone H3 phosphorylation on Ser10 is reduced on the *ixba* promoter when PV is used in presence of TSA (Fig.5D), we checked the recruitment of p65 phosphorylated on Ser276 by ChIP assay (Fig.7C). Whatever the promoter (*ixba* or *icam-1*) and the stimulus (PV or TNFα with or without TSA), the binding of p65-Ser276-P is not significantly modified or slightly reduced after any treatment. The *icam-1* promoter results, shown in Fig.7C, are representatives of the ones obtained on the *ixba* promoter. In summary, TSA addition on PV induction does not influence global ERK and p38 MAPK activation and global phosphorylation of MSK1 targets. This strengthens the idea of a promoter independence after a co-treatment of PV with TSA.

TSA could interfere with p65 transactivation potential depending on the stimulus and the promoter – Then we investigated whether the p65 transactivation potential induced by PV or TNFα is affected by the presence of TSA. The p65-Ser536, included in the transactivation domain, is targeted by multiple kinases (18,19). Its phosphorylation allows Lys310 acetylation by CBP/p300 and is, at least partially, responsible for the p65 transactivation capacity (20). The p65 global phosphorylation status on Ser536 was tested by western blotting on total protein extracts. We observed that the co-stimulation PV plus TSA does not modify the p65 global phosphorylation compared to PV alone (Fig.7D). We then performed ChIP assay to check if the promoter-specific recruitment of p65 phosphorylated on Ser536 could be modified by TSA, even if we observed above no change of the unmodified p65 binding whatever the stimulus and the promoter (Fig.5A and 6A). We first examined the binding on the *ixba* promoter (Fig.8A). The p65-Ser536-P begins to be recruited after 15 min of PV stimulation and a significant reduction of the binding at 30 min is detected in presence of TSA. For TNFα treatment, the addition of TSA leads to an up-regulation of the recruited p65-Ser536-P. The analysis of the *icam-1* promoter revealed that TSA does not modify the PV-induced p65-Ser536-P recruitment at 15 and 30 min of stimulation, but there is a sustained recruitment at 1h when TSA is added (Fig.8B). The TNFα stimulus induces a 3-fold increased binding of p65-Ser536-P at 30 min, which is slightly reduced in presence of TSA. Thus, we can conclude that the p65 transactivation potential on the *ixba* promoter could be affected by the addition of TSA on PV stimulation, partially explaining the delay of *ixba* mRNA expression. This effect is clearly promoter-specific since it is not observed with *icam-1*, another NF-κB-dependent gene.

IKKα recruitment on a defined promoter could be a target for TSA effect depending on the stimulus – Several studies have recently described an important nuclear role for IKKα in transcriptional activation. On one hand, IKKα was shown to phosphorylate histone H3 on Ser10 on the *ixba* promoter which allows subsequent histone H3 acetylation on Lys14 by CBP/p300 (35,38). On the other hand, Mayo and co-workers have highlighted an IKKα-mediated phosphorylation of p65 on Ser536 and SMRT on Ser2410 leading to derepression of SMRT/HDAC3 complexes and p65.
acetylation on Lys310 by CBP/p300 (39,40). Since the influence of the co-treatment of PV with TSA on the delayed \( \text{id}\) mRNA expression appears to result from decreased phosphorylations of both histone H3 on Ser10 and p65 on Ser536, we then wanted to determine whether IKK\( \alpha \) recruitment on \( \text{id} \) promoter could be affected by the addition of TSA on PV stimulation. Indeed, this co-treatment leads to a transient down-regulation of IKK\( \alpha \) binding on the \( \text{id} \) promoter (15 and 30 min), whereas for a stimulation with TNF\( \alpha \) plus TSA, IKK\( \alpha \) recruitment is increased (Fig.8C). We next analyzed \( \text{icam-1} \) promoter and observed that the presence of TSA induces no significant modification after PV induction, while for TNF\( \alpha \), a decrease of IKK\( \alpha \) binding is detected (Fig.8D). Taken together, these results indicate that the delayed \( \text{id} \) mRNA resynthesis, observed when HeLa cells are treated by PV plus TSA, is due to, at least partly, a decreased presence of IKK\( \alpha \). Once again, the nature of NF-\( \kappa \)-B-inducing agent can lead to different response that are promoter specific.

DISCUSSION

Acetylation of histones and transcription factors is tightly regulated by HAT and HDAC. Imbalances of these modifications frequently occur in tumour cells. For instance, abnormal recruitment and activation of HDAC could lead to transcriptional repression of tumour suppressor genes. Therefore, long-time exposure to cells (longer than 24h) with HDAC inhibitors, inducing hyperacetylation, appears to have anti-tumoral effects by reactivating gene expression and altering growth of tumour cells (11,41). Nevertheless, TSA, which is a broad HDAC inhibitor, was previously demonstrated to modify the expression of only 2% of the genes (42). Moreover, among these 2%, genes are up-regulated as well as down-regulated. This highlights the complexity of acetylation phenomenon and the need to further study the regulation mechanisms. Thus, we decided to examine the effect of TSA on the activation of the anti-apoptotic transcription factor, NF-\( \kappa \)-B. Adam et al. have shown that short-time exposure to simultaneous addition of TSA can potentiate TNF\( \alpha \)-induced NF-\( \kappa \)-B activation by, at least partially, extending IKK activity and delaying IkB\( \alpha \) cytoplasmic reappearance (24).

A complementary explanation can come from the fact that HDAC3 is responsible for p65 deacetylation leading to an increased affinity for IkB\( \alpha \) thereby promoting its export to the cytoplasm. Therefore, HDAC inhibition by TSA could impair both IkB\( \alpha \) interaction and nuclear export leading to sustained p65 nuclear residence (43).

In this work, we examined TSA effects on the PV-induced NF-\( \kappa \)-B activation compared to the TNF\( \alpha \)-induced one. The main conclusions are the following. (i) The presence of TSA sustains NF-\( \kappa \)-B activation after each stimulus. This highlights an inhibitory role of HDAC on NF-\( \kappa \)-B activation in both pathways, but by distinct mechanisms. (ii) For PV stimulation, the extension of NF-\( \kappa \)-B activation by TSA could be explained by a delay of \( \text{id} \) mRNA synthesis, whereas, for TNF\( \alpha \), a prolonged IKK activity is expected to be implicated. (iii) The \( \text{id} \) promoter is targeted by the co-treatment PV with TSA, leading to a reduction of RNA Pol II recruitment, histone H3 modifications, p65-Ser536 phosphorylation and IKK\( \alpha \) binding. (iv) According to previous works (35,38,40), we hypothesize, for PV induction, a role of HDAC in the IKK\( \alpha \) recruitment on the \( \text{id} \) promoter. This influences the subsequent histone H3 modifications, RNA Pol II binding but also p65-Ser536 phosphorylation and transactivation via CBP/p300 involvement. (v) TSA effects are clearly promoter-specific as the \( \text{icam-1} \) promoter displays different results.

More precisely, we investigated the impact of TSA on various NF-\( \kappa \)-B signaling pathways such as the classical pathway induced by TNF\( \alpha \) or PMA and the atypical pathways elicited by PV or H\( _{2} \)O\( _{2} \). At first, we demonstrated that TSA acts on NF-\( \kappa \)-B activation in a way depending on the stimulus and the cell type. Indeed, in HeLa cells, TSA prolongs NF-\( \kappa \)-B activation after TNF\( \alpha \), PMA and PV but not after H\( _{2} \)O\( _{2} \). As TSA combined with PMA extends NF-\( \kappa \)-B activation by sustaining the IKK activity, like it was previously demonstrated for TNF\( \alpha \), we did not further study that inductor. The prolonged IKK activity more pronounced with PMA than with TNF\( \alpha \) suggests differences in the implicated pathways that leads to activation/deactivation of the IKK complex. The IKK activation by H\( _{2} \)O\( _{2} \) was slightly decreased by the presence of TSA, but we decided not to further investigate, in this study, this rather modest effect and only...
compare TSA effect after TNFα and PV stimulation. The prolonged NF-κB activation seen with TSA appears to be reproducible to other HDAC inhibitor as well, as it can also be observed with SAHA. Nevertheless, the effect of TSA on PV induction is not general for all cell types. It is very weak in Jurkat T cells of TSA on PV induction is not general for all observed with SAHA. Nevertheless, the effect other HDAC inhibitor as well, as it can also be seen with TSA appears to be reproducible to stimulation. The prolonged NF-κB-induced expression of *ikba* mRNA is not significantly modified by the presence of TSA and the prolonged IKK activity is postulated to be a cause for the delayed IkBa cytoplasmic reappearance (24). For PV stimulation, the addition of TSA does not affect either the IKK or the tyrosine kinase activities but appears to delay *ikba* mRNA synthesis, thereby explaining the prolonged NF-κB residence in the nucleus. Thus, we further analyzed the molecular mechanism of epigenetic modifications responsible for the delayed expression of *ikba* mRNA. We observed a decrease of histone H3 phosphorylation on Ser10, histone H3 acetylation on Lys14 and RNA Pol II recruitment when TSA is added to PV, but not to TNFα. These reductions clarify the delay of *ikba* mRNA synthesis. Recently a nuclear role for IKKα was discovered in histone H3 phosphorylation on Ser10 on the *ikba* promoter, and this phosphorylation appears to be required for subsequent histone H3 acetylation on Lys14 by CBP/p300 (35,38,44). Furthermore, histone H3 must be acetylated to allow accessibility for components of the basal transcriptional machinery, especially RNA Pol II. Thus, if histone H3 phosphorylation is impaired on the *ikba* promoter after co-stimulation PV with TSA, it is likely that CBP/p300 is temporarily impeded and cannot acetylate histone H3 on Lys14 explaining why the RNA Pol II recruitment and *ikba* mRNA synthesis are delayed. Referring to the work of Yamamoto and collaborators, we hypothesize that TSA affects the IKKα activity or its recruitment on the *ikba* promoter leading to a decrease of histone H3 phosphorylation on Ser10 (35). Indeed, an additional ChIP assay (Fig.8C) showed a reduced IKKα recruitment on the *ikba* promoter which could be the cause of the decreased phosphorylation and acetylation of histone H3 and the delay of RNA Pol II recruitment and *ikba* mRNA synthesis.

MSK1 is another histone H3 kinase situated downstream of ERK and p38 in the MAPK pathway (36). The analysis of PV-induced activation of the upstream kinases, ERK and p38 MAPK, and the downstream targets, histone H3 (Ser10) and p65 (Ser276), revealed no modification in presence of TSA, indicating that the global MSK1 activity is not affected. In addition, the binding of p65-Ser276-P on *ikba* and *icam-1* promoters does not appear to be significantly modified after PV or TNFα with or without TSA.

The p65 transactivation potential was also studied through the recruitment of p65 phosphorylated on Ser536 on the *ikba* promoter. We observed that TSA clearly impairs this PV-, but not TNFα-, induced binding, even if a similar amount of p65 is present in the nucleus in these two experimental conditions. This raises the possibility that either phosphorylated p65-S536 could bind to the promoter and be immediately dephosphorylated, or p65 could be phosphorylated on Ser536 after its promoter recruitment. It has been recently demonstrated that chromatin-bound IKKα coordinates simultaneous phosphorylation of p65 on Ser536 and SMRT on Ser2410 on *ciap-2* and *il-8* promoters (40). These phosphorylations deactivate SMRT-HDAC3 repressor complexes and allow p65 to become acetylated on Lys310 by CBP/p300, increasing p65 transactivation. Therefore, we can postulate that the decreased *ikba* mRNA expression after the co-treatment of PV with TSA is a result of a transient impairment of IKKα recruitment on the *ikba* promoter, as it was demonstrated in this study by ChIP assay (Fig.8C). Another argument in favour of the implication of IKKα in our model is that p65 binding on the *ikba* promoter but impair phosphorylation on Ser10 and acetylation on Lys14 of histone H3 (35). Whereas, in IKKβ-/- cells, Hoberg and co-workers have observed a loss of p65 binding on *ciap-2* and *il-8* promoters (39). Of course, we cannot exclude the role of another kinase phosphorylating p65 on Ser536.

Therefore, by the use of the HDAC inhibitor TSA in a PV stimulation context, we postulate that HDAC could have a role in
recruiting IKKα on the *ikba* promoter. Indeed, the presence of TSA to a PV treatment induces an impairment of IKKα binding on the *ikba* promoter, which influences the two following events important for transcription. On one hand, it reduces histone H3 phosphorylation on Ser10, a prerequisite for histone H3 acetylation on Lys14 and RNA Pol II recruitment. And on the other hand, it decreases p65 phosphorylation on Ser536 needed for increasing transactivation via CBP/p300.

Another interesting observation from our study is that the influence of TSA on NF-κB activation seems to be clearly specific of the promoters. We demonstrated that *ikba* and *icam-1* promoters display distinct responses after the co-stimulation by PV or TNFα and TSA with respect to protein recruitments or histone H3 modifications. This is in good agreement with the work of Saccani *et al.* who described the importance of histone H3 phosphorylation for transcription depending on the nature of each promoter (45). In this report, we showed that, for the *icam-1* promoter, the presence of TSA increases the PV-induced histone H3 phosphorylation and IKKα binding, while it reduces the TNFα-one. This situation is opposite to the one observed on the *ikba* promoter. Nevertheless, RNA Pol II recruitment on the *icam-1* promoter does not appear to be modified by TSA addition on PV or TNFα induction. The mechanism on the *icam-1* promoter need to be better understood.

In conclusion, our results suggest that a large range HDAC inhibitor such as TSA is able to influence NF-κB activation in multiple ways. Moreover, an overall role of the HDAC is to inhibit NF-κB activation by different mechanism which depend on the inducer and the considered promoter. This high specificity of NF-κB activation/repression represents an efficient regulation strategy.

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Delay of PV-induced *ikba* mRNA synthesis by TSA addition

REFERENCES

Delay of PV-induced iκbα mRNA synthesis by TSA addition

Delay of PV-induced ikbα mRNA synthesis by TSA addition

FIGURE LEGENDS

Figure 1. Influence of HDAC inhibitor on NF-κB activation and the associated IkBα degradation induced by PV, TNFα, PMA or H2O2 in HeLa cells and Jurkat T cells.

(A-C-D-E) HeLa cells were non treated (NT) or treated with PV (A), TNFα (C), PMA (D) or H2O2 (E) in absence/presence of TSA (A, middle panels-C-D-E) or SAHA (A, right panels) for the indicated times. Nuclear translocation of NF-κB was measured by electromobility shift assay (EMSA) with a probe corresponding to the HIV-1 LTR κB site (upper panels). IkBα degradation was analyzed by western blotting of cytoplasmic extracts (lower panels).

Figure 2. Effect of TSA on IkBα tyrosine phosphorylation or on IKK activity after stimulation by PV, TNFα, PMA or H2O2.

(A) TSA does not modify IkBα tyrosine phosphorylation after PV. IkBα was immunoprecipitated from total cellular extracts obtained after ALLN pretreatment for 45 min and PV with or without TSA for the specified durations. The presence of IkBα phosphorylated on tyrosine in the immunoprecipitated fraction was detected by western blotting with a phospho-tyrosine antibody. Loading control was carried out with an anti-IkBα antibody. (B-D) IKK activity is prolonged by TSA after TNFα or PMA treatment, but not after PV or H2O2 stimulation. (B) Cells were treated with TNFα for 5 min (positive control) or with PV with or without TSA for increasing times. After immunoprecipitation of the complex with an anti-IKKγ antibody, in vitro IKK kinase activity was determined by incubation of the immunoprecipitated proteins with purified GST-IkBα1-54 fusion protein as substrate. A western blotting was then performed using an antibody specific for Ser32-36 phosphorylated IkBα (upper panel). The presence of equal amounts of IKK catalytic subunit (IKKβ) in each sample was confirmed by western blotting (lower panel). (C and D) in vitro IKK kinase activity was measured after treatment with H2O2, TNFα or PMA with or without TSA for different times as described in B.

Figure 3. TSA reduces PV- but not TNFα-induced ikbα mRNA synthesis and NF-κB transcriptional activity.

Total RNAs were isolated at various times of treatment with either PV or PV + TSA (A), and TNFα or TNFα + TSA (B). ikbα mRNA expression level was analyzed by quantitative real-time RT-PCR with specific primers. The results were normalized with the β2-microglobulin transcript. (C) Transient transfection were performed with a luciferase reporter gene under the control of the ikbα promoter. Twenty hours after transfection, cells were treated with PV or TNFα with or without TSA for 8h before cell lysis and detection of luciferase activity, normalized by the protein amount. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments.

Figure 4. TSA decreases TNFα- but not PV-induced icam-1 mRNA synthesis.

Total RNAs were isolated at various times of treatment with either PV or PV + TSA (A), TNFα or TNFα + TSA (B). icam-1 mRNA expression was analyzed by quantitative real-time RT-PCR with specific primers. The results were normalized with the β2-microglobulin transcript. (C) Transient transfection with a luciferase reporter gene under the control of the icam-1 promoter. Twenty hours after transfection, cells were treated with PV or TNFα with or without TSA for 8h before cell lysis and determination of luciferase activity, normalized by the protein amount. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments.

Figure 5. Impact of TSA on p65 and RNA Pol II recruitment and histone H3 modifications on the ikbα promoter after PV or TNFα induction.

ChIP assays were performed on HeLa cells treated with either PV or TNFα with or without TSA using different antibodies for the immunoprecipitation directed against: (A) p65, (B) RNA Pol II, (C) histone H3 acetylated on Lys14 and (D) histone H3 phosphorylated on Ser10. The immunoprecipitated chromatin was submitted to a quantitative real-time PCR analysis using primers amplifying the promoter region of ikbα. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments.
Delay of PV-induced \(ikb\alpha\) mRNA synthesis by TSA addition

**Figure 6.** Impact of TSA on p65 and RNA Pol II recruitment and histone H3 modifications on the icam-1 promoter after PV or TNF\(\alpha\) induction. ChIP assays were performed on HeLa cells treated with either PV or TNF\(\alpha\) with or without TSA with different antibodies for the immunoprecipitation directed against: (A) p65, (B) RNA Pol II, (C) histone H3 acetylated on Lys14 and (D) histone H3 phosphorylated on Ser10. The immunoprecipitated chromatin was submitted to a quantitative real-time PCR analysis using primers amplifying the promoter region of icam-1. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments.

**Figure 7.** Phosphorylation status of ERK and p38 MAPK, histone H3 and p65 after adding TSA to PV induction. (A) ERK and p38 MAPK: HeLa cells were treated with PV or PV with TSA for increasing times. Total proteins were rapidly extracted in SDS-blue lysis buffer and a western blotting was carried out using different antibodies to evaluate ERK and p38 phosphorylation, (B) MSK1 targets: histone H3-Ser10 and p65-Ser276. (C) p65-Ser276-P: ChIP assay was performed on HeLa cells treated with either PV or TNF\(\alpha\) in presence or in absence of TSA with an antibody for the immunoprecipitation directed against p65-Ser276-P. The immunoprecipitated chromatin was submitted to a quantitative real-time PCR analysis using primers amplifying the promoter region of icam-1. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments. (D) p65-Ser536: After PV or PV + TSA treatment, total proteins were rapidly extracted in SDS-blue lysis buffer and a western blotting was carried out using the p65-Ser536-P antibody. For each western blotting (A-B-D), the corresponding loading control was obtained with the antibody directed against unmodified form of the protein.

**Figure 8.** TSA influence on the p65 transactivation potential depends on the stimulus and the promoter. ChIP assays were performed on HeLa cells treated with either PV or TNF\(\alpha\) with or without TSA using different antibodies for the immunoprecipitation directed against: p65 phosphorylated on Ser536 (A-B) and IKKa (C-D). The immunoprecipitated chromatin was submitted to a quantitative real-time PCR analysis using primers amplifying the promoter region of \(ikb\alpha\) (A-C) or icam-1 (B-D). *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments.
Figure 1

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- TSA
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- PMA
- GST-IκBα-P<sub>S32,36</sub>
- IKKβ
Figure 3

A  qRT-PCR $i \kappa b \alpha : PV$

B  qRT-PCR $i \kappa b \alpha : TNF$

C  $i \kappa b \alpha$-luc
Figure 4

A  qRT-PCR icam-1 : PV

B  qRT-PCR icam-1 : TNF

C  icam-1-luc
Figure 5

A. ChIP \( \text{i}k\beta\alpha : p65 \)

B. ChIP \( \text{i}k\beta\alpha : \text{RNA Pol II} \)

C. ChIP \( \text{i}k\beta\alpha : \text{H3-Lys14-Ac} \)

D. ChIP \( \text{i}k\beta\alpha : \text{H3-Ser10-P} \)
Figure 6

A) ChIP icam-1 : p65

B) ChIP icam-1 : RNA Pol II

C) ChIP icam-1 : H3-Lys14-Ac

D) ChIP icam-1 : H3-Ser10-P
Figure 7

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H3-S10-P

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p65-S276-P

p65

C

ChIP icam-1 : p65-Ser276-P

Fold induction

NT  PV 30'  PV 1h  TNF 30'  TNF 1h

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PV

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p65-S536-P

p65
Figure 8

A. ChIP \(iκbα\) : p65-Ser536-P

B. ChIP icam-1 : p65-Ser536-P

C. ChIP \(iκbα\) : IKKα

D. ChIP icam-1 : IKKα

Fold induction

- NT
- PV 15'
- PV 30'
- PV 1h
- TNF 30'
- TNF 1h

+TSA
Histone deacetylase inhibitor trichostatin a sustains sodium pervanadate-induced NF-κB activation by delaying IκBα mRNA resynthesis - comparison with TNFα

Julie Horion, Geoffrey Gloire, Nadia El Mjiyad, Vincent Quivy, Linda Vermeulen, Wim Vanden Berghe, Guy Haegeman, Carine Van Lint, Jacques Piette and Yvette Habraken

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